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Evidence for Apoptosis of Human Macrophage-Like HL-60 Cells by *Legionella pneumophila* Infection

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***Legionella pneumophila*, the causative agent of Legionnaires' disease and Pontiac fever, replicates within and eventually kills human macrophages. In this study, we show that *L. pneumophila* is cytotoxic to HL-60 cells, a macrophage-like cell line. We demonstrate that cell death mediated by *L. pneumophila* occurred at least in part through apoptosis, as shown by changes in nuclear morphology, an increase in the proportion of fragmented host cell DNA, and the typical ladder pattern of DNA fragmentation indicative of apoptosis. We further sought to determine whether potential virulence factors like the metalloprotease and the macrophage infectivity potentiator of *L. pneumophila* are involved in the induction of apoptosis. None of these factors are essential for the induction of apoptosis in HL-60 cells but may be involved in other cytotoxic mechanisms that lead to accidental cell death (necrosis). The ability of *L. pneumophila* to promote cell death may be important for the initiation of infection, bacterial survival, and escape from the host immune response. Alternatively, the triggering of apoptosis in response to bacterial infection may have evolved as a means of the host immune system to reduce or inhibit bacterial replication.**

Legionella pneumophila, the causative agent of Legionnaires' disease, is a facultative intracellular gram-negative bacterial pathogen. The organism survives and multiplies efficiently in monocytes, alveolar macrophages (22), and macrophage-like cell lines such as U937 and HL-60 (28, 33). The pathway for a productive *L. pneumophila* infection begins with the attachment of the bacteria via complement receptors followed by the uptake into the host cell by coiling (20) or conventional phagocytosis. Inside the cell, *L. pneumophila* resides within a specialized phagosome which does not acidify and fails to fuse with primary or secondary lysosomes (19, 21). Over the course of infection, the phagosome surrounds itself with a variety of host cell organelles, such as smooth vesicles, ribosomes, and mitochondria (19, 21). The bacteria are capable of multiplying within the phagosome until the host cell eventually lyses, releasing bacteria to initiate a new infection cycle. Although cytotoxic activity has been reported (15, 26, 34), it is not clear whether it is the action of a specific toxin or simply the large number of bacteria that destroy the cell.

L. pneumophila is known to have cytopathic effects on macrophages, and both intracellular and extracellular (23) bacteria were found to be capable of mediating toxicity. While several potential toxins have been suggested as virulence factors (15, 26, 34), the role of those toxins in the virulence of *L. pneumophila* remains undefined. In fact, there are only a few defined genetic determinants that may be responsible for the cytotoxic effects. A metalloprotease (called ProA or Msp) (35, 38) and a less well defined low-molecular-weight toxin (15) that are cytotoxic for Chinese hamster ovary cells have been described. Both proteins are extracellular factors and have been implicated to act as possible virulence factors causing Legionnaires' disease. A major virulence factor, the Mip (macrophage infectivity potentiator) protein, belongs to the enzyme family of peptidylprolyl *cis/trans* isomerases (14, 18) and was shown to contribute to early survival processes of *L. pneumophila* in phagocytic cells (12).

Necrosis and apoptosis are the two main types of death which can occur in eukaryotic cells. In contrast to necrosis, apoptosis is characterized by the fact that the cell actively takes part in its own death. Necrosis and apoptosis can be distinguished by morphological and biochemical criteria (for a review, see reference 9). Necrosis refers to the morphology most often seen when cells die from severe and sudden injury. In necrosis, there are early changes in mitochondrial shape and function, the plasma membrane is losing its ability to regulate osmotic pressure, and the cell swells and ruptures. Its contents are spilled into the surrounding tissue space and provoke an inflammatory response. The cellular changes in apoptosis are numerous, but it is still not clear which of them are directly associated with death. The plasma membrane becomes ruffled and blebbed, but the cell maintains its osmotic gradient. Furthermore, the cell shrinks remarkably and exhibits an extremely condensed cytoplasma with normal-appearing organelles. The major morphological changes of apoptotic cell death are chromatin condensation and segmentation of the nucleus. The hallmark biochemical feature is the endonuclease-mediated cleavage of internucleosomal DNA linker sections, resulting in the fragmentation of the DNA to multimers of 200 bp (1, 2, 9, 11, 16).

Apoptosis as a response to intracellular infection has been shown for a wide range of pathogens and host organisms (4). The suicide of a pathogen-infected cell may be a productive strategy to guarantee the survival of the multicellular organism, if it succeeds in reducing or eliminating the production of viable pathogenic organisms. In view of the pathogen, death of the host cell may often be required, for example, to allow the eventual release of intracellular organisms.

It has recently been shown that *Shigella flexneri*, an etiological agent of dysentery, *Bordetella pertussis*, the causative agent of whooping cough in humans, and *Actinobacillus actinomycetemcomitans*, an etiological agent in periodontal diseases, induce apoptosis in macrophages and/or macrophage-like cell lines (25, 27, 42). In this study, we report that *L. pneumophila*

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TABLE 1. *L. pneumophila* strains used in this study

Strain[a]	Genotype or description	Reference or source
Corby (S1)	Wild type	24
Philadelphia I (S1)	Wild type	ATCC[b] 33152
JR32	Philadelphia I Sm^r r^- m^+, salt-sensitive isolate	39
Philadelphia I JR32-2	JR32 Δ*mip*::*km*	41
LS2029	Philadelphia I Sm^r Rif^r *lpnR*	29
LS2102	LS2029 *mspA1*::Tn*9*	38

[a] S1, serogroup 1.
[b] ATCC, American Type Culture Collection, Rockville, Md.

can induce apoptosis in the human macrophage-like cell line HL-60. We demonstrate that cell cytotoxicity mediated by *L. pneumophila* occurs at least in part through apoptosis, as shown by changes in nuclear morphology and by DNA fragmentation. Furthermore, we investigated whether either of the *L. pneumophila* genes *mip* and *msp* play a role in the process of inducing apoptosis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this study are described in Table 1. *L. pneumophila* was grown on ABCYE agar plates (13). Antibiotics for *L. pneumophila* selection were kanamycin (50 μg ml^{-1}) and chloramphenicol (5 μg ml^{-1}). Prior to experiments, *L. pneumophila* strains were thinly swabbed on ABCYE plates and grown for 24 to 48 h, when a thin lawn of bacterial growth was visible. Bacteria were harvested from plates, diluted in coincubation medium prior to addition to differentiated HL-60 cells, and stored at −80°C. The number of bacteria was determined by plating 10-fold serial dilutions of bacteria in RPMI 1640 medium (RPMI; Gibco BRL) on ABCYE plates and counting CFU prior to infection in order to monitor an eventual decrease in the CFU.

HL-60 cell culture. The human leukemia cell line HL-60 (10) was maintained in RPMI supplemented with 2 mM L-glutamine (Gln) and 10% fetal calf serum (FCS; Eurobio, Les Ulis, France). HL-60 cells were differentiated into macrophage-like cells by incubating them for 2 days with 10 ng of phorbol 12-myristate 13-acetate per ml (Sigma) in RPMI–2 mM Gln–10% FCS. Adherent cells were washed three times with RPMI–2 mM Gln and then incubated with RPMI–2 mM Gln–10% FCS prior to infection.

Cytotoxicity assay. HL-60 cells were washed in RPMI–2 mM Gln–10% FCS and plated in a 96-well plate at a concentration of 2×10^6 cells per well. *L. pneumophila* was added to the cells at bacterium/cell ratios of 5:1, 50:1, and 500:1. After an incubation time of 2 to 4 days at 37°C under 5% CO_2–95% air, the dye MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] was added to each well at a concentration of 500 μg ml^{-1}. The microtiter dishes were incubated for 4 h at 37°C, the culture medium was aspirated, and the reduced formazan dye was suspended in 100 μl of 0.04 M HCl–1% sodium dodecyl sulfate in isopropanol. The plates were read on a microplate reader (Bio-Rad model 450) at a wavelength of 570 nm. The A_{570} values of eight wells containing the same bacterium/cell ratio were averaged to determine the extent of macrophage killing. The percent cytotoxicity was calculated by the following formula: percent cytotoxicity = 100 × (1 − optical density at 570 nm at 2 to 4 days postinfection/optical density at 570 nm at 3 h postinfection).

DNA fragmentation. Differentiated HL-60 cells (2×10^6 cells per well) were plated in a 24-well plate and infected with *L. pneumophila* strains at a bacterium/cell concentration of 10:1 if not stated otherwise. As a positive control for apoptosis or DNA fragmentation, cells were incubated in RPMI–2 mM Gln–10% FCS containing 1 μg of actinomycin D (ActD; Sigma) per ml. As a negative control, cells were incubated noninfected in RPMI–2 mM Gln–10% FCS and underwent the same treatment as the infected and ActD-treated cells. To assess whether the bacteria have to be viable to induce apoptosis, heat-inactivated bacteria were used for infection as an additional control. Heat inactivation was achieved by heating the bacterial suspension at 95°C for 30 min, and complete inactivation was verified by plating an aliquot on ABCYE. After an incubation time of 24 h at 37°C under 5% CO_2–95% air, the cells were lysed in an equal volume of 2× lysis buffer (0.2% Triton X-100, 10 mM Tris [pH 7.2], 1 mM EDTA [pH 8.0]) and proteinase K (5 mg ml^{-1}). The samples were incubated for 1 h at 50°C and digested with RNase (0.5 mg ml^{-1}). After an incubation time of 1 h at 50°C, the lysates were extracted twice with an equal volume of phenol and once with an equal volume of chloroform-isoamyl alcohol (24:1, vol/vol) before precipitation with ethanol. The precipitates were dried and solubilized in 10 mM Tris (pH 8.0)–1 mM EDTA. Electrophoresis was performed with an 1.8% agarose gel, which was stained with ethidium bromide.

Nuclear staining. HL-60 cells were differentiated in eight-well chamber slides (Nunc) at a concentration of 5×10^5 cells per well. Cells were washed three times in RPMI–2 mM Gln and overlaid with 100 μl of RPMI–2 mM Gln–10% FCS. Cells were infected with *L. pneumophila* strains at bacterium/cell ratios of 1:1, 5:1, 10:1, and 50:1. Positive and negative controls were performed as described above. After an incubation time of 24 h at 37°C under 5% CO_2–95% air, cells were washed three times with prewarmed phosphate-buffered saline (PBS), fixed with 3% paraformaldehyde in PBS, and incubated for 1 h at room temperature (RT) with 20 μl of benzimide trihydrochloride Hoechst 33258 (Sigma) (dilution of 1:500 in PBS). Slides were washed three times with PBS and covered with Mowiol (Hoechst) or Citifluor (Citifluor, London, United Kingdom) to prepare permanent samples. Hoechst 33258-labeled samples were analyzed by fluorescence microscopy at 450 to 490 nm.

Quantitation of apoptotic cells. The percentages of apoptotic cells in the untreated, ActD-treated, and infected HL-60 cell monolayers were assessed by counting the Hoechst 33258-labeled cells under the fluorescence microscope. Ten to sixty visual fields, corresponding to a minimum of 500 cells per experiment, were counted.

RESULTS

Cytotoxic effects of *L. pneumophila* wild-type and mutant strains. The cytotoxicity of *L. pneumophila* to the human macrophage-like cell line HL-60 cells was tested by the MTT viability assay. *L. pneumophila* was able to induce cytotoxicity following 2 days of incubation (Fig. 1). The viability of HL-60 cells infected with *L. pneumophila* Corby at a bacterium/cell ratio of 50:1 was reduced to 82 and 53% after cells were cultured for 2 and 4 days, respectively. *L. pneumophila* Corby killed HL-60 cells effectively, with a maximal cytotoxicity (56.9% ± 1.7%) at a bacterium/cell ratio of 500:1 (Fig. 1).

We compared the cytotoxic activities of *L. pneumophila msp* and *mip* mutant strains (Fig. 2) after 4 days of incubation by the MTT viability assay. All *L. pneumophila* strains used in this study strongly induced cell death at a bacterium/cell ratio of 10:1. However, *L. pneumophila* mutant strains JR32-2 (*mip*) and LS2102 (*msp*) elicited a decreased level of cytotoxicity (62.8 and 43.2%) compared to the wild-type strains Corby and JR32.

DNA fragmentation of macrophage-like HL-60 cells infected with *L. pneumophila* Corby. One of the main characteristics of apoptosis is the fragmentation of the DNA into multimers of 200 bp. To determine whether *L. pneumophila* promoted apoptosis, we examined the induction of DNA fragmentation of HL-60 cells. As shown in Fig. 3 (lanes 3, 4, 7, and 8), a nucleosome pattern of DNA degradation was observed in HL-60

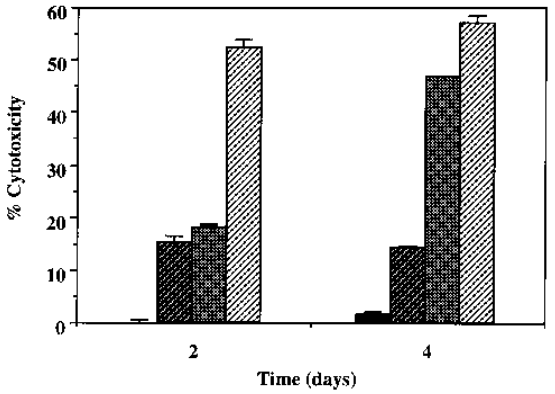

FIG. 1. Cytotoxic effect of *L. pneumophila* Corby (wild type) on differentiated HL-60 cells. HL-60 cells (2×10^6 per well) were infected with *L. pneumophila* Corby at bacterium/cell ratios of 0 (564), 5:1 (▨), 50:1 (▨), and 500:1 (▨). The percent cytotoxicity was determined at 2 and 4 days postinfection by the MTT viability assay as described in Materials and Methods. Data are expressed as the means ± standard deviations of eight replicates. The experiment was performed two times, and similar results were obtained for each experiment.

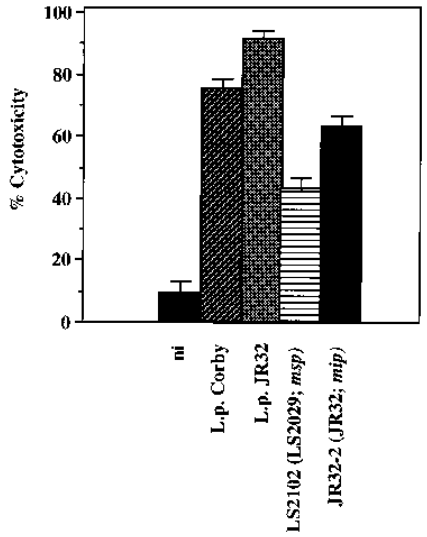

FIG. 2. Cell death of HL-60 cells induced by several strains of *L. pneumophila*. HL-60 cells (2×10^6 per well) were infected with *L. pneumophila* (L.p.) Corby (▨), Philadelphia I JR32 (▩), LS2102 (*mspA*1::Tn*9*) (▭), and Philadelphia I JR32-2 (Δ*mip*::*km*) (▩) at a bacterium/cell ratio of 10:1. Noninfected cells (■) were taken as a control. The percent cytotoxicity was determined at 4 days postinfection by the MTT viability assay as described in Materials and Methods. The ability of strains LS2102 and Philadelphia I JR32-2 to induce cell death is significantly reduced ($P < 0.005$). Data are expressed as the means ± standard deviations of eight replicates. The experiment was performed two times, and similar results were obtained for each experiment.

cells infected with *L. pneumophila* Corby. In fact, the patterns observed for the cellular DNA extracts isolated from cells infected with *L. pneumophila* Corby were similar to those obtained when the cells were treated with ActD (1 μg ml^{-1}) (Fig. 3, lanes 1 and 5), a well-known potent inducer of apoptosis (31). The bands were multimers of 200-bp fragments, indicating internucleosomal fragmentation. The extent of DNA fragmentation increased with the incubation times of 24 h (Fig. 3, lanes 3 and 4) and 48 h (Fig. 3, lanes 7 and 8) and was already detectable at a bacterium/cell ratio of 10:1 (Fig. 3, lanes 3 and 7). This ladder pattern could also be seen after infection with bacteria which had been washed vigorously twice after harvesting and prior to infection (data not shown). This finding rules out the possibility that apoptosis is induced by ingredients of the ABCYE plates rather than the bacteria themselves.

Nuclear morphology of macrophage-like HL-60 cells infected with *L. pneumophila*. Apoptotic cells are characterized by chromatin condensation and segmentation of the nucleus. To investigate the nuclear morphology of macrophage-like HL-60 cells infected with *L. pneumophila* Corby, we stained macrophages with Hoechst 33258, a specific DNA-binding dye. Strong chromatin condensation corresponding to an enhancement of the fluorescence intensity could be observed in HL-60 cells infected with *L. pneumophila* Corby at a bacterium/cell ratio of 10:1 for 24 h (Fig. 4C). A similar morphology was observed in HL-60 cells treated with ActD (1 μg ml^{-1}) for 24 h (Fig. 4B). In contrast, the majority of uninfected cells had a normal healthy appearance and did not show signs of chromatin condensation (Fig. 4A).

As shown in Fig. 5, we were able to detect intracellular bacteria in some of the apoptotic cells. Nevertheless, we cannot exclude the possibility that extracellular bacteria are also able to induce apoptosis, and the relative roles of intracellular and extracellular *L. pneumophila* in triggering apoptosis of the HL-60 cells have to be determined.

Determination of the proportion of apoptotic cells in HL-60 monolayers infected with *L. pneumophila*. To determine the number of apoptotic cells in untreated, ActD-treated, and infected macrophage-like HL-60 cells, DNA was stained with Hoechst 33258 and the number of cells was counted in a microscope. Infection was performed with wild-type Corby at multiplicities of infection (MOIs) of 10 and 100 as well as with heat-inactivated Corby (MOI of 100). As expected from our results with the DNA fragmentation assay, the percentage of naturally occurring apoptotic cells in the untreated HL-60 monolayer is rather high, 23.4% (Fig. 6). The ActD-treated and infected cells display very similar percentages of apoptotic cells: 68.8% for ActD-treated cells and 60.6 and 72.2% for cells infected at MOIs of 10 and 100, respectively. These results are consistent with the results obtained in the DNA fragmentation assay (Fig. 3). In the HL-60 monolayer infected with heat-killed bacteria, the proportion of apoptotic cells was 22.7%, which was similar to the percentage of apoptotic cells in untreated HL-60 cells. Therefore, heat inactivation of the bacteria prior to infection completely destroyed the capability of *L. pneumophila* to induce apoptosis.

DNA fragmentation of HL-60 cells infected with different mutants of *L. pneumophila*. To determine if potential virulence factors like the Mip and Msp proteins are involved in the induction of apoptosis, we tested the induction of DNA fragmentation of HL-60 cells by the parental strains and the corresponding *mip* and *msp* mutant strains. As shown in Fig. 7, the parental strain *L. pneumophila* Philadelphia I JR32 (lanes 7 to 9) as well as the *mip* and *msp* mutant strains (lanes 10 to 12 and 16 to 18) were able to induce DNA fragmentation at 24 h postinfection at a bacterium/cell ratio of 50:1. The patterns observed for the cellular DNA extracts isolated from cells infected with either the parental or mutant strains were similar to that obtained with ActD-treated HL-60 cells (Fig. 7, lanes 1 to 3). Only a weak internucleosomal fragmentation was observed in uninfected HL-60 cells (Fig. 7, lanes 4 to 6) or in HL-60 cells infected with heat-inactivated wild-type bacteria

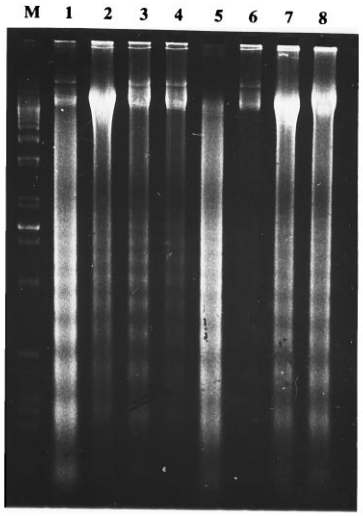

FIG. 3. *L. pneumophila* Corby infection induces internucleosomal DNA cleavage. The cellular DNA was isolated 24 h (lanes 1 to 4) and 48 h (lanes 5 to 8) postinfection from HL-60 cells infected with *L. pneumophila* Corby at bacterium/cell ratios of 10:1 (lanes 3 and 7) and 50:1 (lanes 4 and 8). The DNA samples were analyzed by using an 1.8% agarose gel stained with ethidium bromide. Lane M, molecular size marker (λ, *Hin*dIII); lanes 1 and 5, cellular DNA from ActD-treated (1 μg ml^{-1}) HL-60 cells; lanes 2 and 6, cellular DNA from uninfected HL-60 cells.

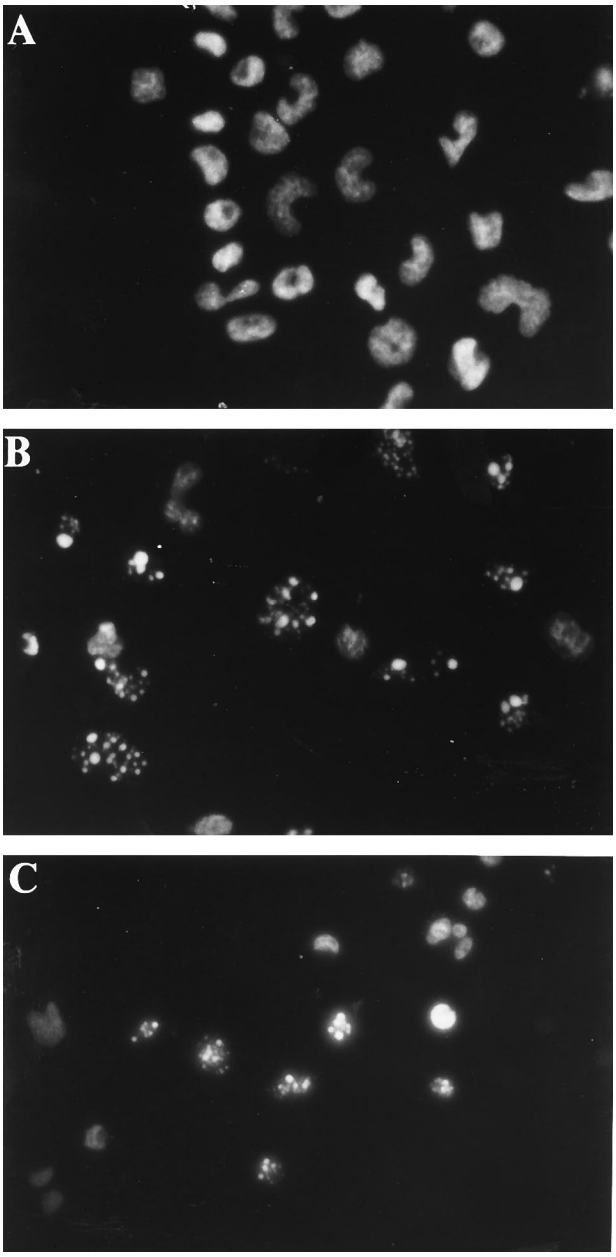

FIG. 4. Nuclear morphology of HL-60 cells infected with *L. pneumophila* Corby. Cells were infected, fixed, and stained with Hoechst 33258. Microscopic observations were made at a magnification of ×63 24 h postinfection. (A) Noninfected cells showing normal chromatin typical for healthy cells; (B) cells treated with ActD (1 μg ml^{-1}), a potent inducer of apoptosis, exhibiting chromatin condensation and nuclear segmentation typical of apoptosis; (C) cells infected with *L. pneumophila* Corby at a bacterium/cell ratio of 10:1 exhibiting apoptotic morphology.

(Fig. 7, lanes 13 to 15). These results demonstrate that neither the Msp nor the Mip protein of *L. pneumophila* is essential for the induction of apoptosis. Furthermore, heat inactivation seems to abolish the capability of *L. pneumophila* to trigger apoptosis. This observation is in good agreement with the data shown in Fig. 6.

DISCUSSION

Bacterial pathogens have developed different strategies to survive inside the host and to overcome natural defenses, thereby causing disease. One strategy is to secrete toxins which efficiently and rapidly destroy the host cells. Another strategy is to invade target cells and to develop intracellularly, avoiding the action of extracellular antibacterial agents. Some bacteria can persist within the phagocytic vacuole, inhibiting phagosome-lysosome fusion or evolving resistance to the acidic environment. Certain invasive pathogens escape from the endocytic vacuole and multiply within the cytoplasm of infected cells.

It has recently been shown that invasive strains of *S. flexneri*, the etiological agent of dysentery, and *A. actinomycetemcomitans*, the etiological agent in periodontal diseases, induce apoptosis in infected macrophages (25, 42). In addition, Khelef et al. reported that *B. pertussis*, the causative agent of whooping cough in humans, induces apoptosis in a murine macrophage cell line and in alveolar macrophages in primary culture (27). This induction is dependent on the expression of adenylate cyclase/hemolysin-encoding gene (27). However, *Listeria monocytogenes*, a gram-positive intracellular pathogen, was shown not to induce apoptosis in murine macrophages (42) but was able to trigger apoptosis in murine dendritic cells, depending on the expression of listeriolysin (17). Therefore, apoptosis is not a general phenomenon of pathogen-infected cells.

L. pneumophila, the pathological agent responsible for Legionnaires' disease and Pontiac fever in humans, multiplies within a specialized phagosome of human macrophages. This phagosome does not acidify and fails to fuse with primary or secondary lysosomes (19, 21). Although cytotoxic effects on macrophages have been reported for both intra- and extracellular bacteria (15, 26, 23, 34), the role of specific toxins in the virulence of *L. pneumophila* remains unclear. However, studies by Sadosky et al. have shown that the ability to kill macrophages seems to be determined by many genetic loci (37). In this study, the human macrophage-like cell line HL-60 was used to assess the cytotoxic properties of *L. pneumophila*, and it was shown for the first time that *L. pneumophila* induced death by apoptosis. *L. pneumophila* Corby killed HL-60 cells effectively, with a cytotoxicity of 18 to 56%, depending on the incubation time and the bacterium/cell ratio used for infection (Fig. 1). Furthermore, we demonstrated that HL-60 cells infected with *L. pneumophila* Corby exhibit a ladder pattern of DNA fragmentation linked to the activation of an internucleosomal nuclease in apoptotic cells (Fig. 3). There is a significant difference in the number of apoptotic cells in infected and untreated HL-60 cells, indicating that the observed cell death is due to *Legionella* infection (Fig. 6). In addition, staining of the nuclei of HL-60 cells infected with *L. pneumophila* Corby showed chromatin condensation and segmentation of the nucleus typical of apoptotic cell death (Fig. 4). The results presented in Fig. 6 and 7 indicate that bacteria must be viable in order to trigger apoptosis in host cells. The presence of extracellular heat-inactivated bacteria and their subsequent phagocytosis seem not to be sufficient for the induction.

Apoptosis is essential for embryogenesis, differentiation, and the control of the immune system (36), and now the importance of apoptosis in infectious diseases is being actively investigated (40). This apoptosis is distinct from accidental cell death or necrosis. The ability of phagocytic macrophages to ingest and kill microorganisms is one of the major mechanisms whereby microbial invasion is prevented by the host. The process of phagosomal acidification by phagosome-lysosome fusion following infection by a microorganism appears to be a crucial step in the degradative process typical for macrophages (21).

Taking these proposals into consideration, we decided to test a few defined mutants of *L. pneumophila* for the ability to

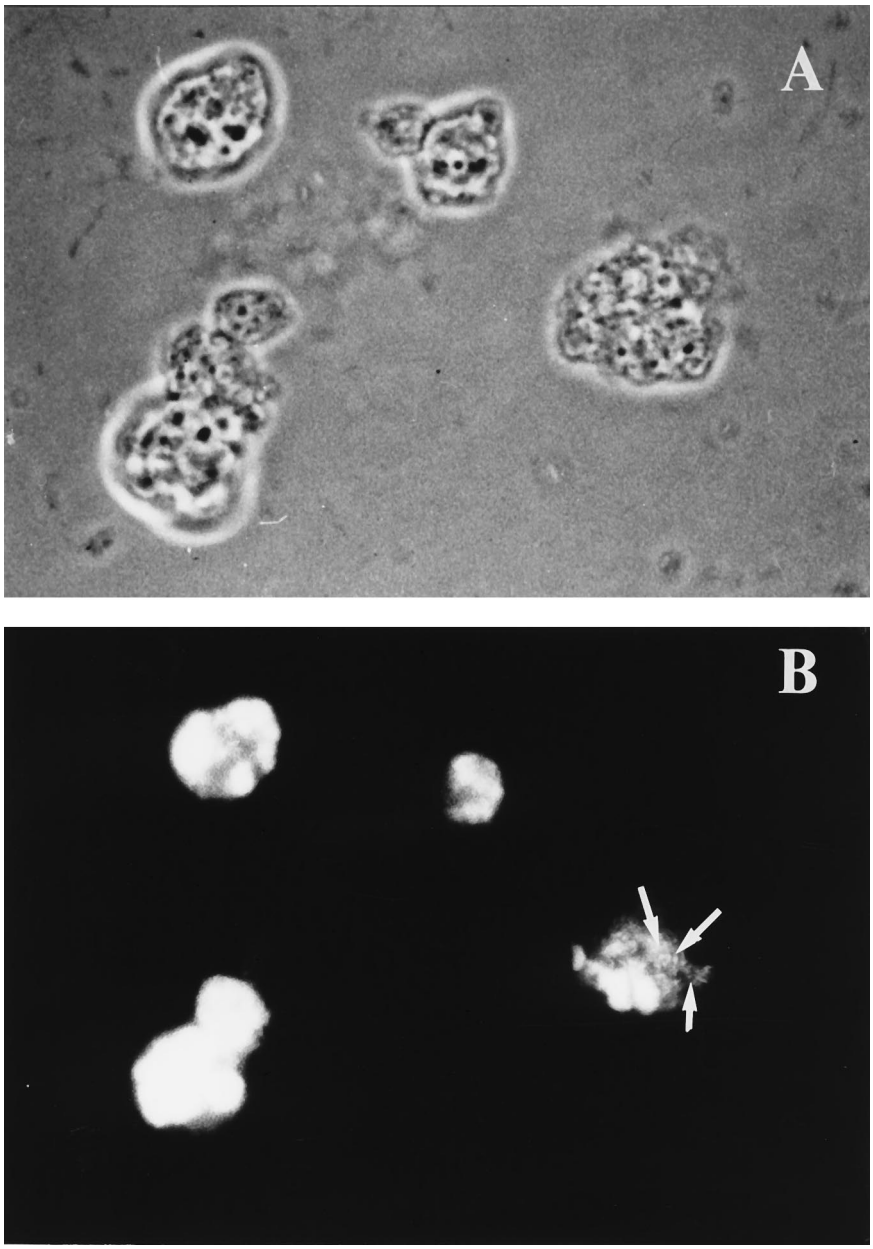

FIG. 5. Intracellular localization of *L. pneumophila* Corby in apoptotic cells. Cells were infected with Corby (MOI of 10), fixed for 1 h with 3% paraformaldehyde, and stained with Hoechst 33258. Microscopic observations were made at a magnification of ×100 24 h postinfection. (A) HL-60 cells in phase contrast; (B) same section as in panel A under 450- to 490-nm fluorescence filter. Apoptotic cells displaying segmented nuclei are entirely filled with bacteria. Intracellular bacteria are indicated by arrows.

induce apoptosis. The Mip protein, a major virulence factor of *L. pneumophila*, contributes to the intracellular bacterial survival in eukaryotic cells and to the in vivo virulence of *Legionella*-infected guinea pigs (5–7). It has recently been shown that Mip belongs to the class of FK506-binding proteins possessing the enzymatic activity of peptidylprolyl *cis*/*trans* isomerases. Full enzymatic activity is not involved in the ability of the bacteria to survive intracellularly (41), which indicates that Mip may have a second, yet undefined function. We demonstrated that a *mip* mutant of *L. pneumophila* is still able to promote apoptosis in HL-60 cells (Fig. 7). Since this mutant strain showed cytotoxic effects in the MTT viability assay (Fig. 2), it seems possible that this virulence factor is involved in cytotoxic mechanisms different from apoptosis.

The major secretory protein (Msp or ProA) is one of the extracellular factors of *L. pneumophila* (35, 38) and has been reported to display cytotoxic and proteolytic activities (3, 34). In fact, there is evidence for the involvement of eukaryotic proteases in triggering apoptosis (32). Proteolytic cleavage of specific substrates may contribute to the process of apoptosis either by structural changes, by activation of other effector molecules, or by removal of an inhibitor. Some of the most characteristic changes associated with apoptosis affect the nucleus and proteases could contribute to DNA fragmentation by activation of endonucleases. At this time, however, the role of these substrates in apoptosis remains largely hypothetical. Nevertheless, these speculations tempted us to examine an *msp* mutant of *L. pneumophila*. Since this mutant strain still pro-

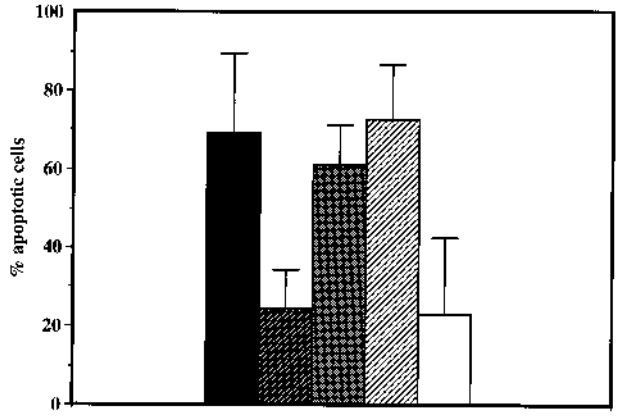

FIG. 6. Percentage of apoptotic cells in untreated, ActD-treated, and Corby-infected HL-60 cells. The cells were incubated for 24 h and stained with Hoechst 33258 as described in Materials and Methods. The percentage of apoptotic cells in each experiment was assessed by counting at least 10 visual fields containing a minimum of 500 cells. The ActD-treated HL-60 culture exhibit 68.8% (■) of apoptotic cells, compared to 23.4% in the untreated HL-60 cells (▨) and 22.7% in HL-60 cells infected with heat-inactivated bacteria (MOI of 100) (□). HL-60 cells infected with wild-type strain Philadelphia I Corby exhibited 60.6% (▩) and 72.2% (▨) of apoptotic cells after infections at MOIs of 10 and 100, respectively. Data are expressed as the mean ± standard deviations of all visual fields. Significant differences in the percentage of apoptotic cells were observed for the infections with wild-type strain Philadelphia I Corby at MOIs of 10 and 100 ($P < 0.001$). Very similar results were obtained in two independent experiments.

motes DNA fragmentation (Fig. 7), Msp may be involved in cytotoxic mechanisms other than apoptosis.

In conclusion, the results presented here support the hypothesis that *L. pneumophila* infection induces an increase in the proportion of fragmented DNA, which correlates with cell death in HL-60 cells. Neither Msp nor Mip is essential for the induction of apoptosis in HL-60 cells. However, they may be involved in other cytotoxic mechanisms that lead to accidental cell death (necrosis).

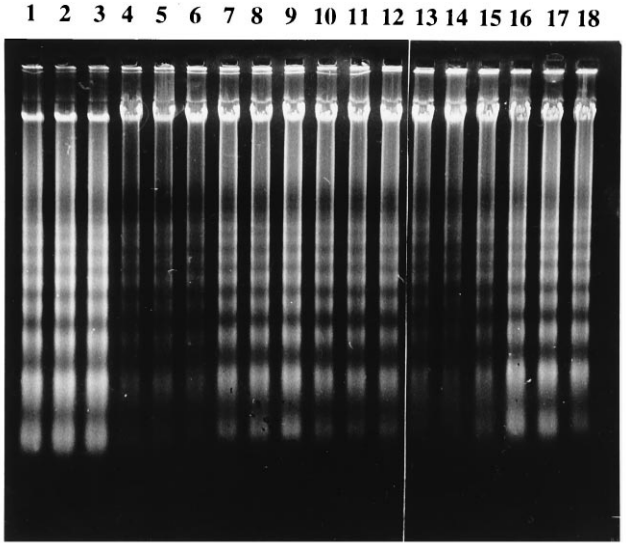

FIG. 7. DNA fragmentation of HL-60 cells infected with different mutants of *L. pneumophila*. Infection and lysis of HL-60 cells, as well as DNA extraction, were done as described in Materials and Methods. HL-60 cells were infected at a bacterium/cell ratio of 50:1 for 24 h. Lanes 1 to 3, cellular DNA from ActD (1 μg ml^{-1})-treated HL-60 cells; lanes 4 to 6, DNA from uninfected HL-60 cells; lanes 7 to 9, DNA from HL-60 cells infected with wild-type strain Philadelphia I JR32; lanes 10 to 12, DNA from HL-60 cells infected with mutant strain JR32-2 (JR32 Δ*mip*::*km*); lanes 13 to 15, DNA from HL-60 cells infected with heat-inactivated wild-type strain Philadelphia I JR32; lanes 16 to 18, DNA from HL-60 cells infected with mutant strain LS2102 (LS2029 *msp*::Tn*9*).

There still remains one basic question: does apoptosis of infected cells benefit the host or the pathogen? There are reasonable arguments for both possibilities: one can imagine that infected cells are sacrificed to protect and maintain the organism on the whole. This hypothesis is supported by the experiments of Molloy et al. (32) comparing the intracellular viability and multiplication of *Mycobacterium tuberculosis* BCG in monocytes pretreated with the chemical apoptosis inducer ATP^{4-} and in untreated monocytes. They demonstrated that apoptotic monocytes inhibit the replication and even reduce the viability of the bacteria compared to nonapoptotic monocytes. Since the infection pathways of mycobacteria and legionellae are very similar (8), these findings may also apply to legionellae.

Considering the pathogen, apoptosis may generate a direct transfer of *L. pneumophila* to a new host cell. Being phagocytosed by intact macrophages while still residing in the apoptotic macrophage, *L. pneumophila* would not have to face the immune response of the host cell.

Further experiments are necessary to determine whether *L. pneumophila* activates the suicide program in the host cell or short-circuits the program by producing a factor that directly causes cell death. Another aspect which needs further investigation is the possible cell specificity of *Legionella*-specific induction of apoptosis. Other host cell systems such as amoebae, human monocytes, and alveolar guinea pig macrophages will be used to verify the presented results in macrophage-like HL-60 cells.

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